Kinetics and control of oxidative phosphorylation in rat liver mitochondria after dexamethasone treatment

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The present investigation was undertaken in order to evaluate the contributions of ATP synthesis and proton leak reactions to the rate of active respiration of liver mitochondria, which is altered following dexamethasone treatment (1.5 mg/kg per day for 5 days). We applied top-down metabolic control analysis and its extension, elasticity analysis, to gain insight into the mechanisms of glucocorticoid regulation of mitochondrial bioenergetics. Liver mitochondria were isolated from dexamethasone-treated, pair-fed and control rats when in a fed or overnight fasted state. Injection of dexamethasone for 5 days resulted in an increase in the fraction of the proton cycle of phosphorylating liver mitochondria, which was associated with a decrease in the efficiency of the mitochondrial oxidative phosphorylation process in liver. This increase in proton leak activity occurred with little change in the mitochondrial membrane potential, despite a significant decrease in the rate of oxidative phosphorylation. Regulation analysis indicates that mitochondrial membrane potential homoeostasis is achieved by equal inhibition of the mitochondrial substrate oxidation and phosphorylation reactions in rats given dexamethasone. Our results also suggest that active liver mitochondria from dexamethasone-treated rats are capable of maintaining phosphorylation flux for cellular purposes, despite an increase in the energetic cost of mitochondrial ATP production due to increased basal proton permeability of the inner membrane. They also provide a complete description of the effects of dexamethasone treatment on liver mitochondrial bioenergetics.

Key words: glucocorticoid, liver, mitochondrion, oxidative phosphorylation efficiency.

INTRODUCTION

Glucocorticoid hormones play an important role in times of stress by regulating salt and water metabolism, blood pressure, immune function, and carbohydrate, protein and fat metabolism. However, chronic glucocorticoid treatment induces side effects, including insulin resistance, a catabolic effect on the skeletal muscle and an increased risk of osteoporosis [1]. It has also been long recognized that the chronic injection of glucocorticoids over 3–7 days decreases the rate of oxygen consumption and lowers P/O ratios of liver mitochondria [2–4]. Such an adverse effect may contribute, at least in part, to the impaired hepatic mitochondrial function of diabetic rats [5]. Interestingly, chronic injection of glucocorticoids does not seem to affect the rate of oxidative phosphorylation of mitochondria from skeletal muscle [6–9], although this may be dependent on the type of fibres contained within the muscle concerned [10] and also on the nature of the substrates oxidized [11]. In essence, these results suggest that chronic treatment with glucocorticoids may depress the efficiency of mitochondrial oxidative phosphorylation in the liver. Nevertheless, a complete description of the effects of chronic glucocorticoid treatment on mitochondrial bioenergetics has not been reported as yet, and the mechanism responsible for the decrease in the metabolic efficiency of liver mitochondria has not been elucidated.

Mitochondrial oxidative phosphorylation plays a central role in metabolism by coupling respiration to the production of ATP. However, this coupling is not perfectly tight, and a portion of mitochondrial respiration is used to compensate for futile proton cycling across the mitochondrial inner membrane via endogenous proton conductance pathways [12]. This cycle is called basal proton leak, and has been estimated to account for approx. 22 % and 34 % of the energy budget of stimulated hepatocytes and contracting skeletal muscles respectively in rats [13]. This proton cycling uncouples mitochondrial respiration from ATP production, thereby lowering the efficiency of oxidative phosphorylation. Therefore, when total mitochondrial respiration is altered, metabolic efficiency will be preserved only if ATP production and proton leak are affected in a similar way. We recently found that 5 days of dexamethasone treatment resulted in an increase in basal proton conductance in the resting state [14] and a decrease in the thermodynamic efficiency of oxidative phosphorylation in liver mitochondria [9]. Such data suggest that chronic glucocorticoid treatment increases proton leakage across the mitochondrial inner membrane, thereby decreasing the efficiency of mitochondrial energy conversion in liver. However, because proton leak and ATP synthesis reactions compete for the same driving force, i.e. the mitochondrial electrochemical proton gradient, it is worth asking whether mitochondrial proton leakage remains significiantly higher under a more physiological state, when proton flux is increased through ATP synthase.

In the present study, we applied top-down metabolic control analysis and its extension, elasticity analysis [15–17], to obtain an overview of mitochondrial processes that play a role in the control and regulation of oxidative metabolism in response to 5 days of dexamethasone treatment. We have also used this description to measure the contributions that ATP synthesis and proton leak reactions may make to the rate of active respiration of mitochondria isolated from the livers of dexamethasone-treated, pair-fed and control rats, when in either a fed or an overnight fasted state.

Abbreviations used: $\Delta\psi$, mitochondrial membrane potential; NEFA, non-esterified fatty acid(s); TPMP, triphenylmethylphosphonium; UCP, uncoupling protein.

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EXPERIMENTAL

Animals

Male Sprague–Dawley rats were caged individually in a temperature-controlled room (22 °C) with a dark/light cycle of 12 h/12 h. They were maintained on a standard rat chow diet consisting of (by weight) 16 % protein, 3 % fat, 60 % carbohydrate and 21 % water, fibre, vitamins and minerals (A04; UAR, Ifacredo, l'Arbresle, France), and were allowed to drink water *ad libitum*. Dexamethasone-treated rats were injected intraperitoneally once daily with dexamethasone (1.5 mg/kg body weight) for 5 days and allowed to feed *ad libitum*. Control rats received no treatment and were fed *ad libitum*. In order to take into account the decrease in food intake induced by dexamethasone treatment [14,18], a third group of pair-fed rats was used. These age- and weight-matched rats were provided with an amount of food equal to the previous day's consumption of the paired dexamethasone-injected rats and were treated with a daily isovolaemic intraperitoneal injection of NaCl (0.9 %) for 5 days. After the final injection of dexamethasone or NaCl, each group (control, pair-fed and dexamethasone-treated) was divided further into two groups, either being provided with food (fed group) or fasted overnight (fasted group) prior to being killed by decapitation.

The present investigation was conducted in accordance with the guiding principles of the French Department of Animal and Environmental Protection for the care and use of laboratory animals.

Analysis of serum NEFA (non-esterified fatty acids)

Blood was collected and centrifuged (1500 ***g***, 10 min, 4 °C). Serum was stored at − 80 °C until assayed. NEFA were determined using a COBAS analyser (Roche Diagnostics, Grenoble, France) with commercially available kits from Boehringer (Grenoble, France).

Liver mitochondria

The liver was immediately dissected and cut up finely with sharp scissors. The minced tissues were diluted 1:10 (w/v) in ice-cold isolation medium containing 100 mM sucrose, 50 mM KCl, 50 mM Tris/HCl and 5 mM EGTA, pH 7.4, at 4 °C and homogenized in a Potter–Elvehjem homogenizer (five passages). The liver homogenate was centrifuged at 800 ***g*** and 4 °C for 10 min. The supernatant was collected, filtered through cheesecloth and centrifuged at 7000 ***g*** for 10 min. The supernatant was discarded, and the pellet was resuspended in isolation medium and centrifuged at 3500 ***g*** for 10 min. Finally, the supernatant was discarded and the pellet resuspended in 1 ml of isolation medium. The protein concentration of mitochondrial suspensions was determined using a bicinchoninic acid assay kit (Interchim, Montluçon, France) with BSA used as standard.

Mitochondrial oxygen consumption rate and $\Delta\psi$ (mitochondrial membrane potential)

These were measured in 3.5 ml incubations in a closed, stirred, Perspex chamber maintained at a temperature of 30 °C using a recirculating water bath and fitted with a Clark oxygen electrode (Rank Brothers Ltd). A combined TPMP (triphenylmethylphosphonium)/reference electrode was fitted through the lid [19]. Liver mitochondria (1 mg of protein/ml) were incubated in an assay medium containing 120 mM KCl, 5 mM KH_2PO_4, 1 mM EGTA, 2 mM $MgCl_2$, 5 μM rotenone, 80 ng/ml nigericin, 0.3 % defatted (w/v) BSA and 3 mM Hepes, pH 7.4. The TPMP electrode was calibrated by sequential 1 μM additions up to 4 μM TPMP, and then 4 mM succinate was added to start the reaction. Finally, 2 μM FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone) was added to dissipate the $\Delta\psi$ and to release all TPMP back into the medium for baseline correction, if needed. The controlled state of respiration (State 4) was measured in the presence of oligomycin (1 μg/ml). The fully active state of mitochondrial respiration (State 3) was recorded with 0.5 mM ADP, which was added prior to calibrating the TPMP electrode. The oxygen solubility of the medium was assumed to be 424.8 nmol of O/ml, and the TPMP binding correction was 0.42 (μl/mg of protein)$^{-1}$ for liver mitochondria [20].

Experimental approach to top-down control analysis

Top-down elasticity analysis, an extension of top-down metabolic control analysis, was used to determine the regulation of oxidative phosphorylation. As described previously [16], we divided the oxidative phosphorylation system into three modules, which are linked by the common intermediate $\Delta\psi$: one $\Delta\psi$ producer [substrate oxidation (S)] and two $\Delta\psi$ consumers [proton leak across the mitochondrial inner membrane (L) and phosphorylation of ADP to ATP (P)]. The kinetics (or elasticity) describing the dependence on $\Delta\psi$ of the fluxes through the substrate oxidation (J_S), leak (J_L) and phosphorylation (J_P) modules were established by carrying out titrations with appropriate inhibitors. The J_S, J_L and J_P fluxes are derived from the oxygen consumption rates driving substrate oxidation, proton leak and phosphorylation respectively. The elasticity of the substrate oxidation module to $\Delta\psi$ ($\varepsilon^{S}{}_{\Delta\psi}$) was calculated from the normalized slope of J_S plotted against $\Delta\psi$ by manipulating the ATP synthesis capacity by the sequential addition of oligomycin from State 3 (oligomycin absent; 500 μM ADP) to the resting state (1 μg/ml oligomycin present). The elasticity of the leak module to $\Delta\psi$ ($\varepsilon^{L}{}_{\Delta\psi}$) was determined from the normalized slope of J_L plotted against $\Delta\psi$ by titrating the electron transport chain with malonate (up to 4 mM) in the presence of oligomycin (1 μg/ml). The elasticity of the phosphorylation module to $\Delta\psi$ ($\varepsilon^{P}{}_{\Delta\psi}$) was determined from the normalized slope of J_P plotted against $\Delta\psi$ by titrating State 3 respiration rate with malonate (up to 250 μM) in the absence of oligomycin, with a saturating amount of ADP (500 μM) present. As total oxygen consumption during State 3 consists of both phosphorylation and leak components, at any given $\Delta\psi$, J_P is calculated as $J_P = J_S - J_L$. The flux control coefficients (C^J), and concentration control coefficients ($C^{\Delta\psi}$) presented in this study were calculated from the elasticities and fluxes as described previously [16], thereby giving a complete description of the internal control of the substrate oxidation, leak and phosphorylation modules over all other modules, as well as $\Delta\psi$.

Effective P/O ratio of mitochondrial oxidative phosphorylation

The oxygen consumed by mitochondria under State 3 respiration is used to drive both ATP synthesis and proton leakage across the mitochondrial membrane. In this context, the efficiency of mitochondrial oxidative phosphorylation (P/O ratio) represents the balance between the rates of ATP synthesis and proton leak, implying that, when total mitochondrial State 3 respiration is altered, efficiency can only be preserved if ATP turnover and proton leak are affected in a similar way. The effective P/O ratios of oxidative phosphorylation in liver mitochondria were calculated as described by Brand et al. [21]. Phosphorylation flux was measured as described above, from the oxygen consumption used to drive phosphorylation (J_P). The effective P/O ratio was subsequently calculated from the flow ratio of J_P divided by the State 3 respiration rate, giving the fraction of oxygen consumption

Table 1 Effects of dexamethasone (Dexa) treatment over 5 days on final body weight, food intake, liver weight and serum NEFA levels

Results are expressed as means ± S.E.M. ($n = 10$). Symbols indicating significant differences ($P < 0.05$) are as follows: *$P < 0.05$ compared with fed rats within the same treatment group; †$P < 0.05$ compared with control rats within the same diet group (fed/fasted); ‡$P < 0.05$ compared with pair-fed rats within the same dietary group. Food intake represents the cumulative food intake for 5 days in each group.

	Fed rats			Overnight fasted rats		
	Control	Pair-fed	Dexa-treated	Control	Pair-fed	Dexa-treated
Final body weight (g)	409 ± 12	360 ± 8†	319 ± 8†‡	362 ± 16*	336 ± 9	292 ± 8*†‡
Food intake (g)	197 ± 8	141 ± 5†	142 ± 6†	163 ± 8*	115 ± 6*†	118 ± 7*†
Liver (g)	17.0 ± 0.5	13.2 ± 0.4†	16.4 ± 0.6‡	10.7 ± 0.3*	9.4 ± 0.2*†	13.9 ± 0.6*†‡
Liver/body weight (g/100 g)	4.2 ± 0.1	3.7 ± 0.1†	5.1 ± 0.1†‡	2.9 ± 0.0*	2.8 ± 0.0*†	4.8 ± 0.1*†‡
Serum NEFA (μM)	337 ± 50	447 ± 56	874 ± 98†‡	658 ± 32*	615 ± 23*	1146 ± 23*†‡

that is effectively used to drive phosphorylation, multiplied by the maximum P/O ratio:

$$\text{Effective P/O ratio} = (\text{P/O})_{\max} \times J_{\text{P}}/\text{State 3 respiration rate}$$

In the present study, we used a $(\text{P/O})_{\max}$ value of 1.5 for the oxidation of succinate [22].

Theory of integrated elasticities and integrated response coefficients [17]

Partial integrated response coefficients describe the extent to which changes in steady-state fluxes and $\Delta\psi$, due to an external effector q, are caused by changes in the substrate oxidation, leak and phosphorylation modules. The fractional change of a flux $[(J_i^{\text{q}} - J_i^{\text{o}})/J^{\text{o}}]$ or $\Delta\psi$ $[(\Delta\psi^{\text{q}} - \Delta\psi^{\text{o}})/\Delta\psi^{\text{o}}]$, in response to an external effector q, was quantified and used to calculate the integrated elasticity of the process i (substrate oxidation, leak or phosphorylation), over the change in the external effector, Δq ($\text{IE}^i{}_{\Delta\text{q}}$). The responses are normalized to the reference state (J^{o} or $\Delta\psi^{\text{o}}$). The activation or inhibition of the process was then calculated as described previously [17]:

$$\text{IE}^i{}_{\Delta\text{q}} = \left[\left(J_i^{\text{q}} - J_i^{\text{o}}\right)/J_i^{\text{o}}\right] - \varepsilon^i{}_{\Delta\psi} \times [(\Delta\psi^{\text{q}} - \Delta\psi^{\text{o}})/\Delta\psi^{\text{o}}]$$

where $\varepsilon^i{}_{\Delta\psi}$ is the elasticity coefficient of module i (substrate oxidation, leak or phosphorylation) to $\Delta\psi$ in the standard state, and superscripts q and o denote their values after and before the addition of effector q (5 days of dexamethasone treatment in the present study).

The flux (C_i^J) and concentration ($C_i^{\Delta\psi}$) control coefficients in the standard state were used to calculate the partial integrated response ($^i\text{IR}_{\Delta\text{q}}{}^{J\text{ or }\Delta\psi}$) of a system flux ($J$) or $\Delta\psi$ when acting through module i (substrate oxidation, leak and phosphorylation):

$$^i\text{IR}_{\Delta\text{q}}{}^{J\text{ or }\Delta\psi} = C_i{}^{J\text{ or }\Delta\psi} \times \text{IE}^i{}_{\Delta\text{q}}$$

Statistical analysis

The statistical significance of observed variations was assessed using two-way ANOVA. Differences between means were subsequently tested by Scheffe's F test. Statistical significance was recognized at $P < 0.05$. Values are presented as means ± S.E.M. The calculation of response coefficients involves combining data from individuals from two different experimental groups (control and dexamethasone-treated); therefore, to avoid data from any single individual biasing our calculations, the raw data from each control animal were compared with those from each dexamethasone-treated animal to generate a series of all possible integrated response coefficients from the empirical data [23]. This set of values was used to test whether a response coefficient was significantly different from zero, thereby representing a real effect.

RESULTS

Body weight, liver weight, food intake and serum NEFA levels

The initial body weight of the rats was 366 ± 5 g, and this did not differ between experimental groups at the beginning of treatment. Regardless of dietary status (fed or overnight fasted), after 5 days of dexamethasone treatment, rats weighed 21% and 12% less than control and pair-fed rats respectively (Table 1). The fact that dexamethasone-treated animals ate the same amount of food as pair-fed animals, but weighed less, indicates that a negative energy balance was more pronounced in rats given glucocorticoids. It was noted that overnight fasting decreased the body weight of rats to the same extent in all experimental groups. When compared with control groups, the liver weights of rats treated with dexamethasone either remained unchanged (fed state) or were increased significantly by 30% (overnight fasted state), depending on dietary status (Table 1). Therefore 5 days of daily dexamethasone injections elicited a significant increase in the liver/body weight ratio in both the fed (+21%) and overnight fasted (+66%) states. Over the same period of time, pair-feeding resulted in a significant decreases in both absolute liver mass and the liver/body weight ratio, irrespective of dietary status (Table 1). The livers of overnight fasted animals showed a significant decrease in absolute weight and in the liver/body weight ratio for all three experimental groups. Liver atrophy was noted to be far less pronounced in dexamethasone-injected rats (−6%) than in their control (−31%) or pair-fed (−24%) counterparts. The current data therefore suggest that dexamethasone treatment counteracts the effects of fasting, as well as of pair-feeding, when considering changes in liver weight.

In fed dexamethasone-treated rats, serum NEFA levels were on average 2.3-fold greater than those of control and pair-fed animals under the same dietary conditions (Table 1). Overnight fasting significantly elevated the levels of circulating NEFA in all three experimental groups, the concentration of which remained significantly higher (less than 2-fold) in dexamethasone-injected rats than in control or pair-fed animals (Table 1).

Mitochondrial substrate oxidation, proton leak and phosphorylation activities

Figure 1 depicts the kinetic responses of the substrate oxidation (Figures 1A and 1B), proton leak (Figures 1C and 1D) and phosphorylation (Figures 1E and 1F) modules to changes in $\Delta\psi$

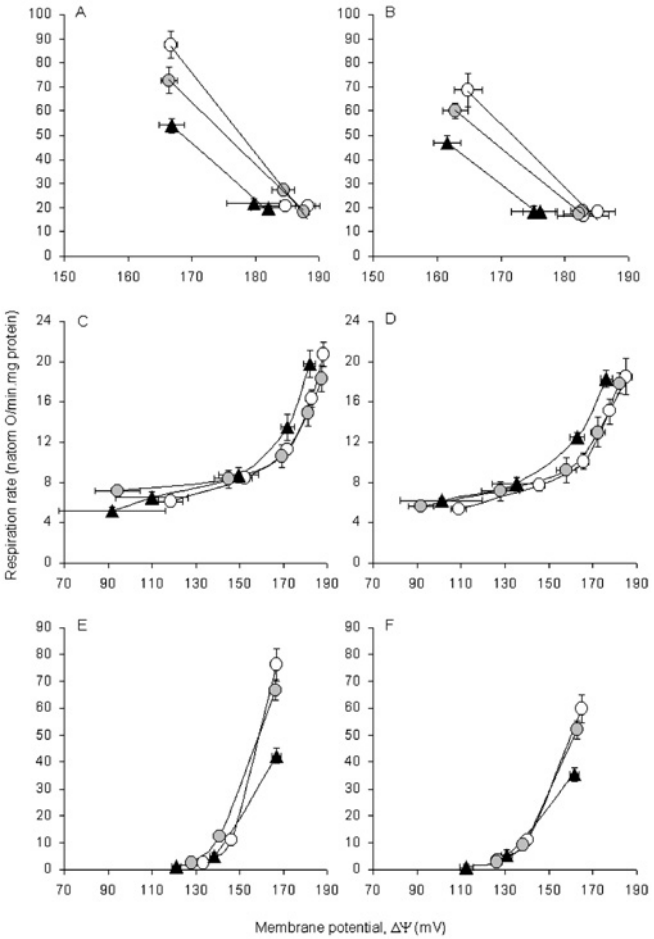

Figure 1 Effects of dexamethasone treatment on the responses of the substrate oxidation, proton leak and phosphorylation modules to $\Delta\psi$

The kinetics of substrate oxidation (**A**, **B**), proton leak (**C**, **D**) and phosphorylation (**E**, **F**) were measured in liver mitochondria respiring on 4 mM succinate from control (○; $n = 6$), pair-fed (shaded circles; $n = 4$) or dexamethasone-treated (▲; $n = 4$) rats in either the fed (**A**, **C**, **E**) or overnight fasted (**B**, **D**, **F**) state. Substrate oxidation kinetics were completed with ADP and succinate and titrated with oligomycin. Proton leak kinetics were completed with succinate and oligomycin and titrated with malonate. Phosphorylation kinetics were completed with ADP and succinate and titrated with malonate. The conditions used for these measurements are described in the Experimental section.

in liver mitochondria from control, pair-fed and dexamethasone-treated animals in either a fed (Figures 1A, 1C and 1E) or an overnight fasted (Figures 1B, 1D and 1F) state. Regardless of dietary status, dexamethasone treatment resulted in a decrease in substrate oxidation activity (Figures 1A and 1B). Indeed, at any given value of $\Delta\psi$, the amount of oxygen used to support the activity of the substrate oxidation reactions was lower in dexamethasone-treated rats than in control or pair-fed animals. Thus the rate of oxygen consumption at maximum rates of ATP synthesis (State 3 respiration; the furthest points to the left in Figures 1A and B) was significantly decreased by an average of 36% and 24% in dexamethasone-treated rats compared with control ($P < 0.05$) and pair-fed ($P < 0.05$) animals respectively. $\Delta\psi$ under State 3 respiration did not differ between groups. Dexamethasone treatment also resulted in increased proton leak activity (Figures 1C and 1D) and decreased phosphorylation activity (Figures 1E and 1F). At any given $\Delta\psi$, the rate of oxygen consumption driving the leak was higher and that driving phosphorylation was lower when compared with observations with

Table 2 Total integrated responses ($IR_{Dexa}^{J or \Delta\psi}$) and integrated elasticities (IE^{i}_{Dexa})

The integrated responses ($IR_{Dexa}^{J or \Delta\psi}$) of substrate oxidation flux, phosphorylation flux, proton leak flux and $\Delta\psi$ to the transition from control to dexamethasone were calculated as $\Delta J/J$ or $\Delta(\Delta\psi)/\Delta\psi$ relative to the standard condition (control rats within the same dietary group). The integrated elasticities (IE^{i}_{Dexa}) of the substrate oxidation, phosphorylation and proton leak modules to dexamethasone were calculated using equations given in the Experimental section. Data are from Figure 1 and Tables 3 and 4. A significant response of the system variable of control rats to dexamethasone treatment is indicated by $^*P < 0.05$.

	$IR_{Dexa}^{J or \Delta\psi}$		IE^{i}_{Dexa}	
Flux or intermediate	Fed rats	Fasted rats	Fed rats	Fasted rats
Substrate oxidation	$-0.37 \pm 0.03^*$	$-0.26 \pm 0.05^*$	$-0.35 \pm 0.05^*$	$-0.38 \pm 0.09^*$
Phosphorylation	$-0.44 \pm 0.03^*$	$-0.36 \pm 0.05^*$	$-0.44 \pm 0.03^*$	$-0.25 \pm 0.04^*$
Leak	0.17 ± 0.07	$0.27 \pm 0.06^*$	0.15 ± 0.07	$0.40 \pm 0.06^*$
$\Delta\psi$	0.00 ± 0.01	-0.02 ± 0.01	–	–

mitochondria of control or pair-fed animals, particularly at higher potentials. Maximal leak-dependent respiration (State 4 respiration measured in the presence of oligomycin; furthest points to the right in Figures 1C and 1D) did not differ between groups. $\Delta\psi$ was, however, slightly decreased in mitochondria from dexamethasone-treated compared with control animals in either the fed ($P = 0.08$, Figure 1C) or overnight fasted ($P < 0.05$, Figure 1D) state. Respiratory control ratios for the control, pair-fed and dexamethasone-treated rats were 4.2 ± 0.1, 4.0 ± 0.1 and 2.7 ± 0.1 respectively in the fed state, and 3.7 ± 0.1, 3.4 ± 0.1 and 2.6 ± 0.2 respectively following an overnight fast. Thus, regardless of dietary status, respiratory control ratios were decreased significantly by glucocorticoid treatment.

Interestingly, pair-feeding tended to decrease the activity of the substrate oxidation module (Figures 1A and 1B), but had no effect on the proton leak and phosphorylation activities (Figures 1C–1F) when compared with corresponding control kinetics. Although these results are in accordance with previously published data from rats undergoing short-term caloric restriction [24], the effects of food restriction on mitochondrial substrate oxidation, proton leak and phosphorylation kinetics may depend on the duration and intensity of the restriction, and may also be tissue- and/or age-specific [23,25–27]. In the present study, we have chosen to focus on the effects of dexamethasone treatment in an attempt to elucidate its mechanism of action, and therefore have not explored further the effects of caloric restriction.

Integrated response coefficients

Table 2 shows the integrated elasticities and overall fractional changes (total integrated responses) of mitochondrial fluxes through the substrate oxidation, phosphorylation and proton leak modules for control rats who underwent dexamethasone treatment ($IR_{Dexa}^{J or \Delta\psi}$). The integrated elasticities of substrate oxidation and phosphorylation were significantly different from zero, suggesting that both were targets for dexamethasone treatment under our experimental conditions. The partial integrated response coefficients showed that the decreases in the rates of State 3 respiration (J_S) and phosphorylation (J_P) induced by dexamethasone treatment (IR_{Dexa}^{J} values were negative; Table 2) were mediated by decreases in the activities of the substrate oxidation and phosphorylation modules (Figure 2, bars a, b, e and f), in both fed and fasted rats.

The integrated responses and elasticities of the proton leak were significant in the overnight fasted state ($P < 0.05$), but failed to reach statistical significance in the fed state ($P = 0.06$), indicating

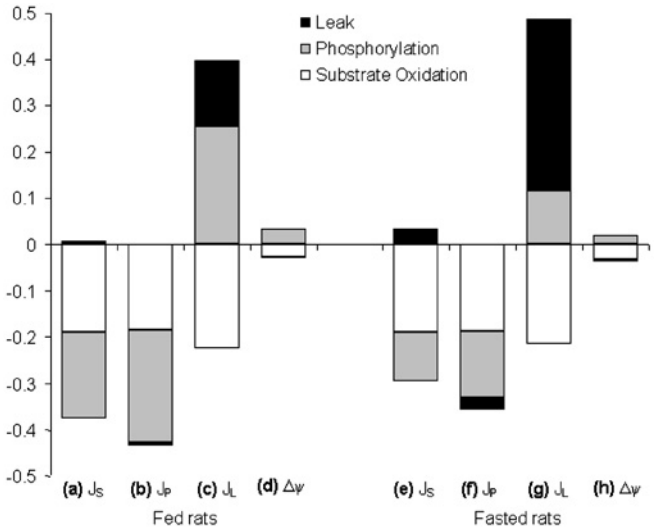

Figure 2 Partial integrated responses of mitochondrial fluxes and $\Delta\psi$ to dexamethasone treatment

Division of the integrated responses to dexamethasone treatment of substrate oxidation rate J_S (a, e), phosphorylation rate J_P (b, f), proton leak rate J_L (c, g) and $\Delta\psi$ (d, h) into partial integrated responses via the substrate oxidation, phosphorylation and proton leak modules is shown. Liver mitochondria were isolated from dexamethasone-treated or control rats in either the fed or overnight fasted state.

that on the whole the proton leakage in mitochondria from dexamethasone-treated rats was higher than that seen in those from control animals (Table 2). The partial integrated responses show that this increase was mediated through the phosphorylation and proton leak modules (Figure 2, bars c and g). This increase was partly counteracted by a simultaneous decrease in the activity of the substrate oxidation module (Figure 2, bars c and g). The State 3 $\Delta\psi$ value was not affected by dexamethasone treatment in the fed or the overnight fasted state ($IR_{Dexa}{}^{\Delta\psi}$ values were not different from zero; Table 2). Such homoeostasis resulted from the fact that the partial responses via substrate oxidation and phosphorylation were equal and opposite (Figure 2, bars d and h).

Efficiency of mitochondrial oxidative phosphorylation

We determined the effective P/O ratios of mitochondria isolated from the livers of control, pair-fed and dexamethasone-treated rats in either the fed or the overnight fasted state (Figure 3A). Regardless of dietary status, the effective P/O ratio of liver mitochondria was decreased significantly by 12% following 5 days of dexamethasone injections. Consequently, the fraction of mitochondrial respiration devoted to proton leak was significantly increased in the livers of rats given glucocorticoids as compared with their control and pair-fed counterparts (Figure 3B).

Relationship between serum NEFA levels and mitochondrial efficiency

A significant inverse correlation was observed between serum NEFA levels and the effective P/O ratio (Figure 4A), which was attributed to the amount of oxygen consumed to drive phosphorylation (Figure 4B), and not that devoted to proton leakage (Figure 4C). In addition, the serum NEFA level was related to State 3 respiration ($r = -0.72$, $P < 0.0001$), but not to State 4 respiration ($r = -0.10$, $P = 0.59$). Consequently, we also observed a significant inverse correlation between the serum NEFA level and the respiratory control ratio ($r = -0.82$, $P < 0.0001$).

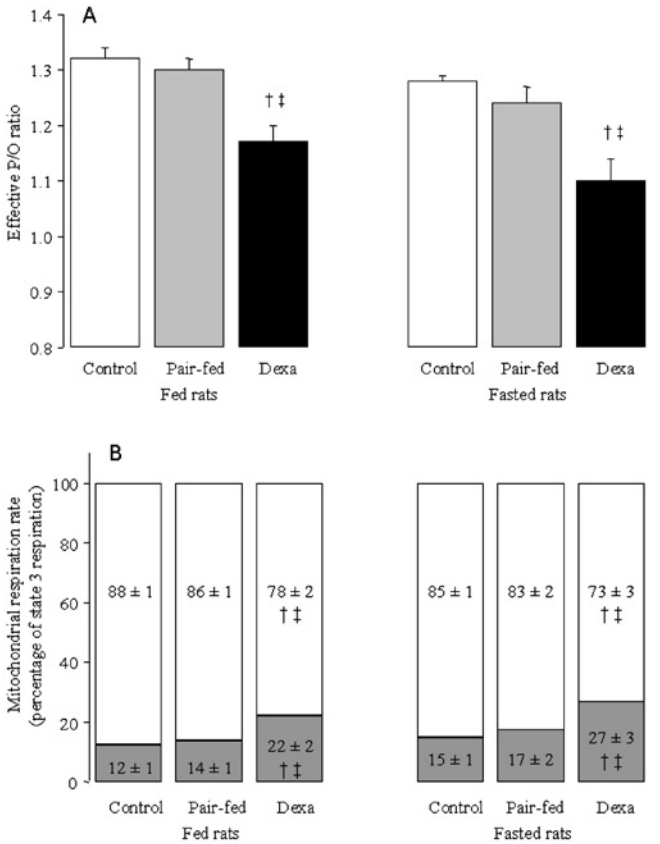

Figure 3 Effects of dexamethasone treatment on effective P/O ratios and on the contributions of ATP turnover and proton leak to mitochondrial State 3 respiration

Liver mitochondria were isolated from control ($n = 6$), pair-fed ($n = 5$) or dexamethasone (Dexa)-treated rats ($n = 5$) in either the fed or overnight fasted state. Succinate (4 mM) in the presence of rotenone (5 μM) was used as the substrate; see the Experimental section for details. (**A**) Effective P/O ratios of phoshorylating mitochondria in control, pair-fed and dexamethasone groups. (**B**) Respiration driving proton leak (grey bars) and respiration driving ATP turnover (white bars). Symbols indicate significant differences ($P < 0.05$) as follows: †, difference from control within the same dietary group (fed/fasted); ‡, difference from pair-fed within the same dietary group.

Metabolic control analysis

Table 3 gives the elasticities of the substrate oxidation, phosphorylation and proton leak modules to $\Delta\psi$. Elasticity coefficients describe the extent to which a small change in $\Delta\psi$ can affect the rate through each module. Regardless of dietary status, dexamethasone treatment elicited a slight, statistically insignificant 20–30% increase in the elasticity values of the substrate oxidation module within this group, compared with either the control or pair-fed groups. Dexamethasone treatment tended to lower the elasticity values of the phosphorylation and proton leak modules, although this failed to reach statistical significance for the proton leak module when compared with control values in either the fed ($P = 0.08$) or overnight fasted ($P = 0.12$) rats. However, pair-feeding induced similar changes in the elasticity values of both the phosphorylation and proton leak modules when compared with control values, with the exception of the elasticity of the phosphorylation module in overnight fasted rats (Table 3).

Flux control coefficients describe the extent to which small changes in the activities of the modules affect the flux through each module, and are summarized in Table 4. Dexamethasone treatment

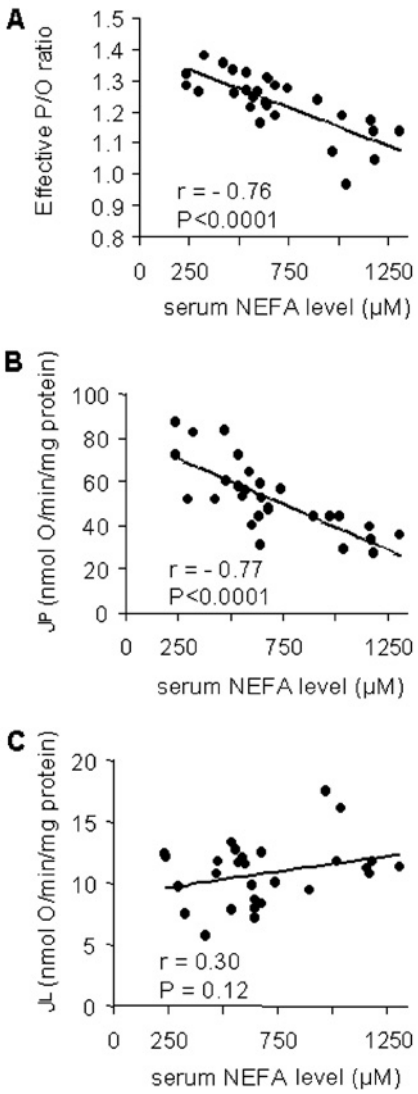

Figure 4 Relationships between serum NEFA levels and mitochondrial efficiency

Correlations of the levels of serum NEFA with effective P/O ratio (**A**), phosphorylation rate (**B**) and proton leak rate (**C**) of phosphorylating mitochondria are shown.

Table 3 Effects of dexamethasone (Dexa) treatment on the elasticities of the substrate oxidation, phosphorylation and proton leak modules to $\Delta\psi$

S, P and L denote the substrate oxidation, phosphorylation and proton leak modules respectively; ε, elasticity. Results are means ± S.E.M. ($n = 4$–6). Symbols indicate significant differences as follows: $^*P < 0.05$ compared with control within the same dietary group (fed/fasted); †$P < 0.05$ compared with pair-fed within the same dietary group.

	Fed rats			Overnight fasted rats		
	Control	Pair-fed	Dexa-treated	Control	Pair-fed	Dexa-treated
$\varepsilon^{S}_{\Delta\psi}$	−5.9 ± 0.4	−5.9 ± 0.7	−7.2 ± 0.4	−6.3 ± 0.4	−5.7 ± 0.2	−7.6 ± 0.8
$\varepsilon^{P}_{\Delta\psi}$	6.3 ± 0.3	5.0 ± 0.1*	4.9 ± 0.2*	5.5 ± 0.2	5.1 ± 0.0	4.4 ± 0.2*†
$\varepsilon^{L}_{\Delta\psi}$	7.8 ± 0.7	4.6 ± 0.7*	5.7 ± 0.7	6.6 ± 0.6	4.2 ± 0.5*	5.1 ± 0.6

resulted in significant changes in the control of each module over their own rate (C_S^{JS} and C_L^{JL} were decreased; C_P^{JP} was increased). In addition, the control exerted by substrate oxidation activity over the phosphorylation flux (C_S^{JP}) was decreased, and the control coefficient of the leak module over flux through the substrate oxidation module (C_L^{JS}) was increased. All these effects were related to the hormone treatment, as the corresponding control coefficients were not altered by pair-feeding. Table 4 also shows that dexamethasone treatment elicited significant changes in the control coefficients of the proton leak (C_L^{JP}) and phosphorylation (C_P^{JL}) modules over phosphorylation and proton leak fluxes respectively. This can be ascribed to the decrease in food intake by dexamethasone-treated rats rather than to a specific effect of glucocorticoids.

Concentration control coefficients of the three modules over $\Delta\psi$ are summarized in Table 4. Regardless of dietary status, the control exerted by both $\Delta\psi$ consumers [phosphorylation ($C_P^{\Delta\psi}$) and proton leak ($C_L^{\Delta\psi}$)] over the level of $\Delta\psi$ was affected by dexamethasone treatment. However, the effect of glucocorticoid on $C_P^{\Delta\psi}$ failed to reach statistical significance when dexamethasone values were compared with fed ($P = 0.07$) and overnight fasted ($P = 0.09$) control values.

Table 4 also shows the control coefficients of the substrate oxidation, phosphorylation and proton leak modules over the effective P/O ratio ($C^{P/O}$). As originally reported by Brand et al. [21], the effective P/O ratio was largely controlled by both the phosphorylation and proton leak modules, with substrate oxidation having a lower degree of control. The phosphorylation module had greater positive control in the mitochondria of dexamethasone-treated rats than in those of the corresponding control and pair-fed animals, irrespective of dietary status. Dexamethasone treatment also induced an increase in negative control by the proton leak module of the effective P/O ratio as compared with mitochondria from control and pair-fed rats. It should be noted that this failed to reach statistical significance only when it was compared with the value for mitochondria from overnight fasted pair-fed rats ($P = 0.08$). No differences were observed between the control coefficients of the substrate oxidation modules of mitochondria from dexamethasone-treated and control rats. When compared with control conditions, pair-feeding decreased the small negative control that substrate oxidation had on the effective P/O ratio (Table 4).

DISCUSSION

The primary question addressed in the present study is whether or not glucocorticoids affect the contribution that the mitochondrial proton cycle makes to the rate of oxidative phosphorylation in active mitochondria. These results show that the chronic injection of dexamethasone for 5 days resulted in a decrease in the effective P/O ratio, in accordance with previously reported decreases in the efficiency of the mitochondrial oxidative phosphorylation process in rats given glucocorticoids [2–4,9]. This implies that the fraction of respiration devoted to proton leakage in phosphorylating liver mitochondria is increased [21], a condition in which ATP synthesis has been maximally stimulated. This increase was shown to be specific to the glucocorticoid treatment, because no change was noted in liver mitochondria from pair-fed rats. These findings are of fundamental importance to our understanding of cell energetics in glucocorticoid-induced hypermetabolism, because a change in the efficiency of working mitochondria must affect cellular energy wastage. Our results suggest that the energetic cost of mitochondrial ATP production is increased in the livers of rats given dexamethasone, the liver being an organ in which glucocorticoids have been demonstrated to stimulate energy-demanding processes such as gluconeogenesis and ureagenesis [28,29].

Table 4 Flux control coefficients over substrate oxidation, phosphorylation and leak fluxes, and concentration control coefficients over $\Delta\psi$, in liver mitochondria from control, pair-fed and dexamethasone (Dexa)-treated rats

J_S, J_P and J_L denote substrate oxidation, phosphorylation and proton leak fluxes respectively. Results are expressed as means ± S.E.M. ($n = 4$–6). Symbols indicate significant differences as follows: *$P < 0.05$ compared with control within the same dietary group (fed/fasted); †$P < 0.05$ compared with pair-fed within the same dietary group.

	Fed rats			Overnight fasted rats		
Control coefficient	Control	Pair-fed	Dexa-treated	Control	Pair-fed	Dexa-treated
Control coefficient over oxidation flux						
C_S^{JS}	0.52 ± 0.02	0.46 ± 0.03	0.41 ± 0.02*	0.47 ± 0.02	0.46 ± 0.01	0.38 ± 0.03*†
C_P^{JS}	0.42 ± 0.02	0.46 ± 0.03	0.45 ± 0.01	0.45 ± 0.02	0.44 ± 0.01	0.45 ± 0.02
C_L^{JS}	0.06 ± 0.01	0.08 ± 0.01	0.14 ± 0.02*†	0.08 ± 0.01	0.10 ± 0.01	0.18 ± 0.02*†
Control coefficient over phosphorylation flux						
C_S^{JP}	0.51 ± 0.02	0.47 ± 0.03	0.40 ± 0.01*	0.46 ± 0.03	0.48 ± 0.02	0.37 ± 0.02*†
C_P^{JP}	0.55 ± 0.2	0.60 ± 0.03	0.69 ± 0.01*†	0.61 ± 0.02	0.61 ± 0.02	0.74 ± 0.02*†
C_L^{JP}	−0.06 ± 0.01	−0.07 ± 0.01	−0.09 ± 0.01*	−0.07 ± 0.00	−0.09 ± 0.01*	−0.10 ± 0.02*
Control coefficient over leak flux						
C_S^{JL}	0.63 ± 0.04	0.44 ± 0.07*	0.47 ± 0.07	0.55 ± 0.04	0.40 ± 0.04*	0.43 ± 0.07
C_P^{JL}	−0.55 ± 0.04	−0.37 ± 0.06*	−0.37 ± 0.07*	−0.47 ± 0.03	−0.32 ± 0.03*	−0.32 ± 0.06*
C_L^{JL}	0.93 ± 0.01	0.94 ± 0.01	0.90 ± 0.01*†	0.92 ± 0.01	0.93 ± 0.01	0.89 ± 0.01*†
Concentration control coefficients over $\Delta\psi$						
$C_S^{\Delta\psi}$	0.08 ± 0.00	0.09 ± 0.01	0.08 ± 0.00	0.08 ± 0.00	0.09 ± 0.00	0.08 ± 0.01
$C_P^{\Delta\psi}$	−0.07 ± 0.00	−0.08 ± 0.01	−0.06 ± 0.00†	−0.07 ± 0.00	−0.08 ± 0.00	−0.06 ± 0.01†
$C_L^{\Delta\psi}$	−0.01 ± 0.00	−0.01 ± 0.00	−0.02 ± 0.00*†	−0.01 ± 0.00	−0.02 ± 0.00*	−0.02 ± 0.00*
Control coefficients over P/O ratio						
$C_S^{P/O}$	−0.07 ± 0.01	−0.03 ± 0.02*	−0.06 ± 0.03	−0.05 ± 0.01	−0.01 ± 0.01*	−0.04 ± 0.03
$C_P^{P/O}$	0.18 ± 0.00	0.17 ± 0.01	0.28 ± 0.00*†	0.20 ± 0.01	0.19 ± 0.02	0.31 ± 0.02*†
$C_L^{P/O}$	−0.11 ± 0.01	−0.14 ± 0.01	−0.22 ± 0.03*†	−0.14 ± 0.01	−0.18 ± 0.02*	−0.27 ± 0.04*

Interestingly, we observed that the effective P/O ratios and rates of phosphorylation of liver mitochondria were negatively related to serum NEFA levels. There is evidence that the degree of coupling in liver mitochondria is dependent on the tissue fatty acid concentration, which is likely to be dictated by the corresponding concentration in the plasma [30,31]. It has been reported that infusion of fatty acids into the perfused liver resulted in a decline in the P/O ratio [32]. Others have reported that liver mitochondria isolated from fasted rats undergoing a single bout of treadmill exercise exhibited lower efficiency of oxidative phosphorylation [33,34], which was not directly related to plasma NEFA [34]. However, since these functional alterations were reversed in the presence of BSA and were mimicked by adding small amounts of NEFA to isolated mitochondria [34], it remains uncertain whether this uncoupling arose from the contamination of isolated mitochondria by NEFA during the isolation procedure. In the present study, mitochondria were incubated in the presence of 0.3 % (w/v) BSA to prevent such NEFA-induced uncoupling. The question therefore arises as to how serum NEFA could ultimately influence mitochondrial function in the liver. One hypothesis involves the regulation of mitochondrial metabolism by the cellular accumulation of their associated CoA thioesters [35], which have been consistently found to inhibit carriers of several mitochondrial metabolites, such as the adenine nucleotide translocator, the phosphate carrier and the dicarboxylate carrier [36]. Another hypothesis is that mitochondrial coupling and enzymic activity are regulated by reversible fatty acylation of some mitochondrial proteins [37–39]. Yet another possibility is that hepatic long-chain fatty acylcarnitines might simply be incorporated into the mitochondrial membranes, thereby altering their fluidity [40] and in turn the mobility of electron transport chain components within the membrane. Although we cannot substantiate any of these hypotheses, it should be noted that we have found a strong and highly significant relationship between the level of serum NEFA and the effective P/O ratio in isolated liver mitochondria.

Regulation analysis indicated that the increase in the rate of respiration due to proton leak, in response to dexamethasone treatment, is mediated through an increased activity of the proton leak pathways as well as a decrease in the activity of the phosphorylation module (as the phosphorylation module had a negative control coefficient over proton leak flux; see Table 4 and [16,20, 23,26]). However, this increase in mitochondrial proton leakage was blunted by the tendency of the simultaneous decrease in the activity of the substrate oxidation module to lessen proton leakage, due to its positive control coefficient over proton leak flux (Table 4 and [16,20,23,26]). These effects on the substrate oxidation and proton leak modules explain further why dexamethasone treatment resulted in a small decrease in $\Delta\psi$ under State 4 conditions (oligomycin present), while basal oxygen consumption remained unchanged. A decrease in substrate oxidation activity means that fewer protons will be pumped out of the mitochondrial matrix by the electron transport chain, causing $\Delta\psi$ to fall and oxygen consumption to decrease. In contrast, an increase in proton leak activity means that more protons will return from the intermembrane space into the matrix, causing $\Delta\psi$ to fall and oxygen consumption to increase. An increase in proton leak flux that is cancelled out by a decrease in substrate oxidation flux explains why the oxygen consumption under State 4 remained unchanged.

As mentioned above, the increase in the activity of basal proton leakage in liver mitochondria proved to be specific to dexamethasone treatment, as no changes in proton leak kinetics were noted in mitochondria from pair-fed rats in either the fed or the overnight fasted state. To date, the only proven protein uncouplers of oxidative phosphorylation are the UCPs (uncoupling proteins). Within this protein family, UCP1 is the only member that catalyses

significant basal proton leakage in isolated mitochondria, but this protein is expressed exclusively in the brown adipose tissue of mammals. The other members of the mammalian UCP family, UCP2 and UCP3, catalyse a GDP-sensitive inducible proton leak in the presence of superoxide or peroxidation derivative products, such as reactive aldehydes [41–43], but there is no evidence to indicate that these proteins are constitutively active in liver mitochondria [41]. Similarly, the adenine nucleotide translocator has been demonstrated to catalyse inducible proton conductance in the presence of 4-hydroxy-2-nonenal [42], a prominent product of lipid peroxidation [44]. Nevertheless, it has been shown that mitochondria respiring on succinate in the presence of the complex I inhibitor, rotenone (the experimental condition used in the present study), do not generate measurable reactive oxygen species [45,46], and therefore it is unlikely that the increase in the rate of basal proton leak was due to oxidative stress-induced uncoupling activity through the adenine nucleotide translocator or even through UCP-dependent pathways. The adenine nucleotide translocator and other members of the mitochondrial carrier family have also been reported to catalyse a fatty acid-dependent proton conductance [47]. Given that glucocorticoid treatment is known to induce an increase in liver fatty acids [48], the question of whether such uncoupling could arise from the contamination of isolated mitochondria by fatty acids during the isolation procedure should be asked. In the present study, because of the presence of 0.3 % (w/v) BSA in the respiratory medium, this is unlikely to have been the case. Alternatively, the increase in the proton leak activity of mitochondria from glucocorticoid-treated rats could be the result of an increase in the inner membrane surface area, changes in fatty acid composition, or a change in some unknown mitochondrial membrane protein that catalyses a proton leak. As yet the precise effects of glucocorticoids on mitochondrial proton conductance are unclear, but they may be tissue specific [14].

The decrease in the rate of mitochondrial phosphorylation (J_P) due to dexamethasone treatment, despite a simultaneous increase in the uncoupled respiration of phosphorylating mitochondria, seems to contradict the fact that glucorticoids increase cellular ATP demand in the liver [28,29]. In this respect, metabolic control analysis performed in the present study tells us how such an unfavourable energetic profile in isolated mitochondria may not necessarily be associated with impaired mitochondrial ATP production in the liver. We found that the internal control coefficients of the mitochondrial phosphorylation module over the rate of phosphorylation and the effective P/O ratio were both increased following dexamethasone treatment. This implies that any stimulation of phosphorylation activity, which can result from increased cellular ATP consumption and demand, will increase both the rate of mitochondrial ATP synthesis and oxidative phosphorylation efficiency. A further insight is that dexamethasone treatment resulted in a decrease in the internal control coefficient of the substrate oxidation module over phosphorylation flux. This implies, for instance, that an increase in the activity of substrate oxidation following increased substrate delivery to the mitochondria would not result in an increase in mitochondrial ATP flux in the livers of dexamethasone-treated rats compared with controls. It therefore appears that stimulation of cellular ATP-consuming reactions with glucocorticoids is the only way in which the liver can overcome the decrease in the mitochondrial phosphorylation flux.

Another important result is that the State 3 $\Delta\psi$ value was not altered by dexamethasone treatment, despite a decrease in the mitochondrial oxidative phosphorylation rate. Regulation analysis indicated that this high degree of homoeostasis for $\Delta\psi$ [49,50] is the consequence of equal inhibition of the mitochondrial supply and demand reactions in rats given dexamethasone. The decrease in substrate oxidation activity induced by dexamethasone treatment had a tendency to decrease $\Delta\psi$ (as the substrate oxidation module had a positive control coefficient over $\Delta\psi$; see Table 4 and [16,20,23,26]). This decrease was in turn exactly opposed by a simultaneous decrease in the activity of the phosphorylation module, which increased $\Delta\psi$, due to its negative coefficient over $\Delta\psi$ (Table 4 and [16,20,23,26]). Such $\Delta\psi$ homoeostasis is of importance, because it allows phosphorylating mitochondria to maintain a constant transport of ions and metabolites across the mitochondrial membrane, or likewise a constant phosphorylation potential, in order to fulfil cellular energetic demands.

In conclusion, it is apparent that dexamethasone is a modulator of mitochondrial bioenergetics in the liver. Indeed, the present findings show that 5 days of dexamethasone treatment specifically increased the contribution of the mitochondrial proton cycle to the oxidative phosphorylation rate of active mitochondria in the liver. This increase in the fraction of uncoupled respiration was achieved with no change in $\Delta\psi$. We also found that dexamethasone treatment strengthened the internal control of the phosphorylation module (including the ADP/ATP translocator, the phosphate carrier and ATP synthase) over mitochondrial ATP synthesis. Given these results, we propose that phosphorylating liver mitochondria in glucocorticoid-treated rats can maintain ATP flux for cellular needs, despite an increase in the energetic cost of mitochondrial ATP production because of increased basal proton permeability of the inner membrane. Hence stimulation by glucocorticoids of the metabolic rate in the liver will increase whole-body energy expenditure, and thereby contribute to the negative energy balance observed in these animals.

This work was supported by a grant from 'contrat de region Pays de Loire et Bretagne'. D. R. was supported by a grant from 'conseil général de Maine et Loire'. We thank Antoine Augeraud for technical assistance, Miriam Ryan for linguistic improvements, and Pierre Legras, Jérôme Roux and Dominique Gilbert for animal care.

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Received 28 April 2004/25 May 2004; accepted 3 June 2004
Published as BJ Immediate Publication 3 June 2004, DOI 10.1042/BJ20040696